Connexins Act as Tumor Suppressors in Three-dimensional Mammary Cell Organoids by Regulating Differentiation and Angiogenesis

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Abstract

Connexins are tumor suppressors, and human breast connexin 26 (Cx26) and connexin 43 (Cx43) gap junctions are often down-regulated in breast cancer. We previously showed that Cx26 and Cx43 overexpressed in MDA-MB-231 breast cancer cells inhibited tumor growth *in vivo* but not in two-dimensional cultures. In the current study, we show that overexpression of Cx26 or Cx43 has tumor-suppressive properties in a three-dimensional environment such that they reduced anchorage-independent cell growth and induced partial redifferentiation of three-dimensional organoids of MDA-MB-231 cells. Importantly, the majority of exogenous connexins did not localize to the cell-cell interface or rescue gap junctional intercellular communication (GJIC) as assessed by dye transfer, providing evidence of a GJIC-independent mechanism of mammary tumor suppression. To further elucidate the mechanisms involved in connexin-induced three-dimensional redifferentiation of tumor cells, we examined whether connexin expression has a role in epithelial to mesenchymal transition (EMT). Cx26 and Cx43 reduced cell migration, increased cytokeratin 18 expression, and decreased vimentin levels, indicating a shift from a mesenchymal towards an epithelial phenotype. In addition, we examined the role of connexins in angiogenesis by probing an angiogenesis antibody array with conditioned media from three-dimensional MDA-MB-231 cultures. This revealed that connexin overexpression regulated various angiogenesis-linked proteins. Furthermore, secreted factors from connexin overexpressing cells inhibited endothelial cell tubulogenesis and migration, and xenografts of Cx43 overexpressing MDA-MB-231 cells showed reduced tumor angiogenesis. In summary, Cx26 and Cx43 inhibit the malignant properties of MDA-MB-231 cells via GJIC-independent mechanisms, including regulation of EMT and angiogenesis. (Cancer Res 2006; 66(20): 9886-94)

Introduction

In the normal human mammary gland, the gap junction proteins connexin 43 (Cx43) and connexin 26 (Cx26) are expressed. Cx43 is not only predominantly expressed in myoepithelial cells but also in luminal cells of the ducts and the secretory epithelium and at myoepithelial-luminal cell interfaces (1, 2). Cx26 is expressed in luminal epithelial cells of the alveoli (3). In human breast cancer tissues, Cx43 gap junctions are commonly down-regulated compared with the nonneoplastic breast tissue surrounding primary tumors (2). Furthermore, both Cx26 and Cx43 expression are down-regulated in many mammary carcinoma cell lines, suggesting that gap junctions play a role in maintaining cell differentiation and preventing transformation (4–7). Conversely, when connexins are overexpressed in carcinoma cells, tumor growth is slowed, and the cells regain the capacity to form at least partially differentiated structures (8). Additionally, mice lacking Cx32 are highly susceptible to liver and lung tumor formation (9–12), leaving little doubt that connexins exhibit tumor-suppressive properties.

MDA-MB-231 cells are a mammary tumor cell line that expresses no Cx26 and only modest amounts of Cx43 and exhibit low levels of gap junctional intercellular communication (GJIC) compared with normal breast epithelial cells. Our previous studies have shown that retroviral delivery of Cx26 or Cx43 to MDA-MB-231 cells did not substantially increase GJIC or suppress growth in two-dimensional culture; however, it did result in growth suppression *in vivo* (13). Consequently, the mechanism by which Cx26 or Cx43 are invoking tumor suppression may not be strictly limited to events related to GJIC, and due to its lack of notable GJIC rescue, this cell model is ideal to test this hypothesis.

MDA-MB-231 cells can successfully be cultured in reconstituted extracellular matrix; thus, we have chosen to examine the mode(s) of connexin tumor suppression in three-dimensional organoids to more closely mimic the architecture of the *in vivo* environment. We show that both Cx26 and Cx43 inhibit the malignant phenotype of MDA-MB-231 cells in the absence of rescuing gap junction-mediated dye transfer, possibly by interfering with processes involved in epithelial to mesenchymal cell transition and angiogenesis. We further show that these findings are not restricted to MDA-MB-231 cells by employing a second breast tumor cell line (MDA-MB-435) that is connexin deficient.

Materials and Methods

Cell culture. MDA-MB-231 and MDA-MB-435 human breast tumor cells (American Type Culture Collection, Manassas, VA) were cultured in RPMI 1640 or high glucose DMEM (Invitrogen, Burlington, Ontario, Canada), respectively, supplemented with 10% fetal bovine serum, 100 units/mL of penicillin, and 100 μg/mL of streptomycin at 37°C with 5% CO_2. Cx43- and Cx26-overexpressing MDA-MB-231 and MDA-MB-435 cells were used as previously described (13, 14). MCF-10A cells (American Type Culture Collection), a nontumorigenic epithelial cell line, were cultured in Mammary Epithelial Growth Medium (Clonetics-Cambrex, Guelph, Ontario, Canada) supplemented with a BulletKit (CC-3150) and 100 ng/mL of cholera endotoxin (Sigma, Oakville, Ontario, Canada). For three-dimensional cultures, cells were plated in a 1:1 dilution of Growth Factor Reduced Matrigel (BD Biosciences, Mississauga, Ontario, Canada) at 5×10^4/mL and

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overlaid with culture medium. Cultures were grown for 7 to 10 days before morphologic analysis or protein/RNA isolation. Human microvascular endothelial cells (HMVEC; Clonetics) and human endothelial cells from umbilical vein (HUVEC; Clonetics) were cultured in EGM-2 or EBM-2 media, respectively, supplemented with a BulletKit (CC-3162).

Soft agar assay. MDA-MB-231 cells were plated in 0.3% agar at 4×10^4/mL, and after 10 days of culture, colony formation (clusters of $\geq$10 cells) in each cell line was scored from 10 low-power microscope fields taken with differential interference contrast microscopy (DIC) on a Zeiss (Thornwood, NY) axioscope. The number of colonies from three independent experiments was summed and averaged for statistical analysis using the Student's *t* test.

Three-dimensional morphogenesis assay. Ten fields of each well of MDA-MB-231 cells were imaged at low power with DIC on a Zeiss axioscope. Colony morphogenesis was quantified by counting the number of spherical versus stellate colonies within each image. A colony was deemed stellate if it had one or more off-shoot from the central sphere of cells. Four independent trials were summed for percentage and SD calculations before statistical analysis using the Student's *t* test.

Immunocytochemistry. Fixed three-dimensional cultures of MDA-MB-231 cells were double-labeled for connexins and a variety of resident marker proteins. Control and Cx43-expressing cells were labeled with either a polyclonal anti-Cx43 (1:400; Sigma) or a noncommercial monoclonal anti-Cx43 in blocking solution. Cx26-expressing cells were labeled with noncommercial polyclonal or monoclonal anti-Cx26 antibody. An FITC-conjugated anti-rabbit or anti-mouse IgG (1:200; Jackson ImmunoResearch, West Grove, PA) was used as the secondary antibody for connexin labeling. For immunolocalization of marker proteins, cells were labeled with a monoclonal anti-vimentin antibody (1:200; Chemicon, Temecula, CA), a polyclonal anti-cytokeratin 18 antibody (1:200; Calbiochem, San Diego, CA), a polyclonal anti-type IV collagen antibody (1:100; Rockland, Gilbertsville, PA), or a monoclonal anti-β_1 integrin antibody (1:50; Chemicon). Texas Red–conjugated anti-rabbit or anti-mouse IgG (1:200; Jackson ImmunoResearch) was used as the secondary antibody. Cell cultures were then stained with Hoechst 33342 (1:1,000; Molecular Probes, Eugene, OR). Labeled cultures were imaged on a Zeiss LSM 510 META inverted confocal microscope mounted with a $\times$40 or $\times$63 oil (1.4 numerical aperture) objective (15). Where expression level comparisons were being made between control and connexin-expressing cells, the imaging variables were kept constant between samples.

Fluorescence recovery after photobleaching analysis. Three-dimensional cell cultures were loaded with 5 ng/μL of calcein-AM (Molecular Probes) and 10 μg/mL of Hoechst 33342, for nuclear staining, in an isotonic glucose solution for 15 minutes at room temperature. An image was taken in a focal plane within the middle of an organoid, and then a small area of cells on the edge of the organoid was photobleached with intense laser scanning (488 nm). After ensuring that the entire z-plane of the organoid was fully bleached, images were recorded from the original focal plane at 1-minute intervals for 10 minutes to monitor the recovery of gap junction-mediated dye diffusion into the bleached area. Three-dimensional cultures of MCF 10A cells that express functional Cx43 and Cx26 were used as a positive control.

MDA-MB-231 migration. To assess cell migration, 1×10^3 cells were plated on top of FluoroBlok Transwell (BD Biosciences) filters. After 24 hours, cells were fixed in hemacolor fixative solution (EM Science, Gibbstown, NJ) and stained with 0.1% Hoechst 33342, and the number of cells on the bottom and top of the filters was counted for 10 microscope fields using OpenLab software (Improvision, Lexington, MA). The results are presented as the ratio of cells that migrated over 24 hours to the bottom of the filter versus the sum of cells on the top and bottom. SDs across all trials were calculated, and statistical significance was determined using a Student's *t* test.

Western blot analysis. Cell lysates (10-20 μg) from three-dimensional MDA-MB-231 cell cultures or two-dimensional MDA-MB-435 cell cultures was separated by electrophoresis on 10% SDS-PAGE gels, transferred to nitrocellulose membranes, and immunoblotted with a vimentin-specific monoclonal antibody (1:5,000; Chemicon) or an anti-cytokeratin 18 antibody (1:1,000; Calbiochem). Antibody labeling was detected using the enhanced chemiluminescence system (Pierce, Rockford, IL). The membranes were reprobed for glyceraldehyde-3-phosphate dehydrogenase (1:10,000; Chemicon) as a control for normalized protein loading. Densitometry was done using KODAK 1D (Toronto, Ontario, Canada) software, and the relative intensities of the bands were statistically analyzed using a Student's *t* test and represent the average $\pm$ SD of at least three independent Western blots.

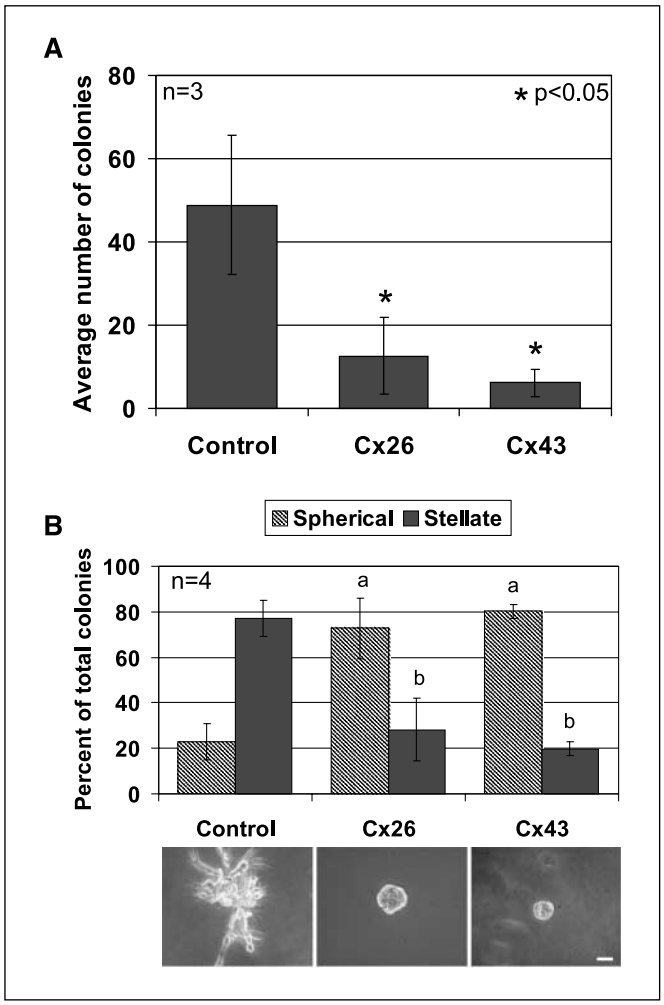

Figure 1. Connexin overexpression reduces anchorage-independent growth and induces partial organoid redifferentiation in three-dimensional culture. *A,* cells overexpressing either Cx26 or Cx43 formed significantly less colonies in soft agar than control cells. *B,* MDA-MB-231 organoid shape was scored as either stellate (malignant) or spherical (re-differentiated) after growth in Matrigel. Seventy-seven percent of control cells formed stellate colonies, whereas the majority of Cx26- or Cx43-overexpressing cells (73% and 80%, respectively) formed spherical organoids. a, $P < 0.05$, significant difference from the control spherical value; b, $P < 0.05$, significant difference from the control stellate value. Phase-contrast images are of representative three-dimensional organoids. Bar, 50 μm.

Reverse transcription-PCR. Total RNA was isolated from three-dimensional cultures with Trizol reagent (Invitrogen) according to protocol. The RNA was then diluted to equal concentration for all samples, and semiquantitative reverse transcription-PCR (RT-PCR) was carried out with the QIAGEN One-step RT-PCR kit (Mississauga, Ontario, Canada) using the following primers: MCP-1, forward 5′-GCCTTAAGTAATGTTAATTCTTAT-3′; MCP-1, reverse 5′-GGTGTAATAGTTACAAAATATTCA-3′; interleukin-6 (IL-6), forward 5′-CCTTCCAAAGATGGCTGAAA-3′; IL-6, reverse 5′-AGCTCTGGCTTGTTCCTCAC-3′; β-actin, forward 3′-AAGAGAGGCATCCTCACCCT-5′; β-actin, reverse 3′-TACATGGCTGGGGTGTTGAA-5.

All products were assayed in the logarithmic phase of the RT-PCR cycles and compared with β-actin for template control by gel electrophoresis and ethidium bromide visualization. Experiments were repeated from three independent sets of cell cultures.

Angiogenesis antibody array. To assess if molecules important in angiogenesis were regulated by cells overexpressing connexins, the RayBio Human Angiogenesis Antibody Array I.I (RayBiotech, Inc., Norcross, GA) was screened according to protocol. One milliliter of serum-free medium conditioned for 48 hours by three-dimensional cultures of control MDA-MB-231 cells and Cx26- or Cx43-overexpressing cells was used to probe the array. Two (Cx26) or four (Cx43) sets of arrays were probed with conditioned medium from independent sets of three-dimensional cultures. Densitometry and statistical analysis were done as for the immunoblots normalizing to the positive and negative controls on the array.

Tubulogenesis assay. A thin layer of Matrigel in a 12-well plate was overlaid with 5×10^4 HMVEC or HUVEC. The cells were allowed to seed for 2 hours before the EGM-2 medium was removed and replaced with 0.5 mL serum-free medium conditioned for 48 hours by confluent two-dimensional cultures of control or connexin-overexpressing MDA-MB-435 or MDA-MB-231 cells or 7- to 10-day-old three-dimensional cultures of MDA-MB-231 cells. As a further control, one well of cells was incubated in fresh endothelial cell medium. After 16 to 48 hours of growth, tube formation was quantified. Ten fields were imaged from each well of endothelial cells with DIC on a Zeiss axioscope, and the degree of tubulogenesis was then quantified from the images by calculating the area covered with cells within each image using the Pathology Picture Analysis System (Jiyisoftware.com, Xuzhou, China). Results from independent trials were summed for the average and SD calculations before statistical analysis using the Student's *t* test.

HUVEC migration. The lower chambers of precoated (0.15% gelatin) FluoroBlok Transwell (BD Biosciences) filters were filled with 600 μL of serum-free medium conditioned for 48 hours by confluent two-dimensional cultures of control or connexin-overexpressing MDA-MB-435 or MDA-MB-231 cells or 7- to 10-day-old three-dimensional cultures of MDA-MB-231 cells. HUVEC were suspended in EBM2 media, and 1×10^4 cells (in 100 μL) were inoculated into the upper chamber of each Transwell. After 24 hours of incubation, the cells were fixed in hemacolor fixative solution (EM Science) and stained with 0.1% Hoechst 33342, and the number of cells on the bottom and top of the filters was counted for 10 microscope fields using OpenLab software (Improvision). The results are presented as the percentage of cells that migrated to the bottom of the filter versus the sum of cells on the top and bottom.

Analysis of *in vivo* tumor angiogenesis. Sections of xenograft tumors in mammary fad pads of nude mice formed of viral vector control or Cx43-overexpressing MDA-MB-231 cells were previously prepared (13). Ten microscope fields of a section of each xenograft were examined, and the number of vessels present in tumor tissue was calculated.

Results

Connexin overexpression reduces the malignant properties of breast tumor cells. MDA-MB-231 cells were grown in soft agar to assess their capacity for anchorage-independent growth, which is a key feature of cell transformation. Cells overexpressing either Cx26 or Cx43 showed significantly decreased colony formation compared with control cells expressing only an empty vector (Fig. 1*A*). Similar results were seen in a mildly aggressive variant of MDA-MB-435 cells expressing both functional and nonfunctional Cx26 species (16). These results suggest that overexpression of Cx26 or Cx43 partially inhibits breast tumor cell transformation. Due to the importance of cell-cell and cell-matrix interactions (17), we hypothesized that reinstatement of a three-dimensional environment may be essential to assess the mechanism of connexin-based tumor suppression. To that end, MDA-MB-231 cells and connexin-expressing variants were grown in three-dimensional cultures to evaluate their growth and differentiation properties. The majority of control MDA-MB-231 cells formed

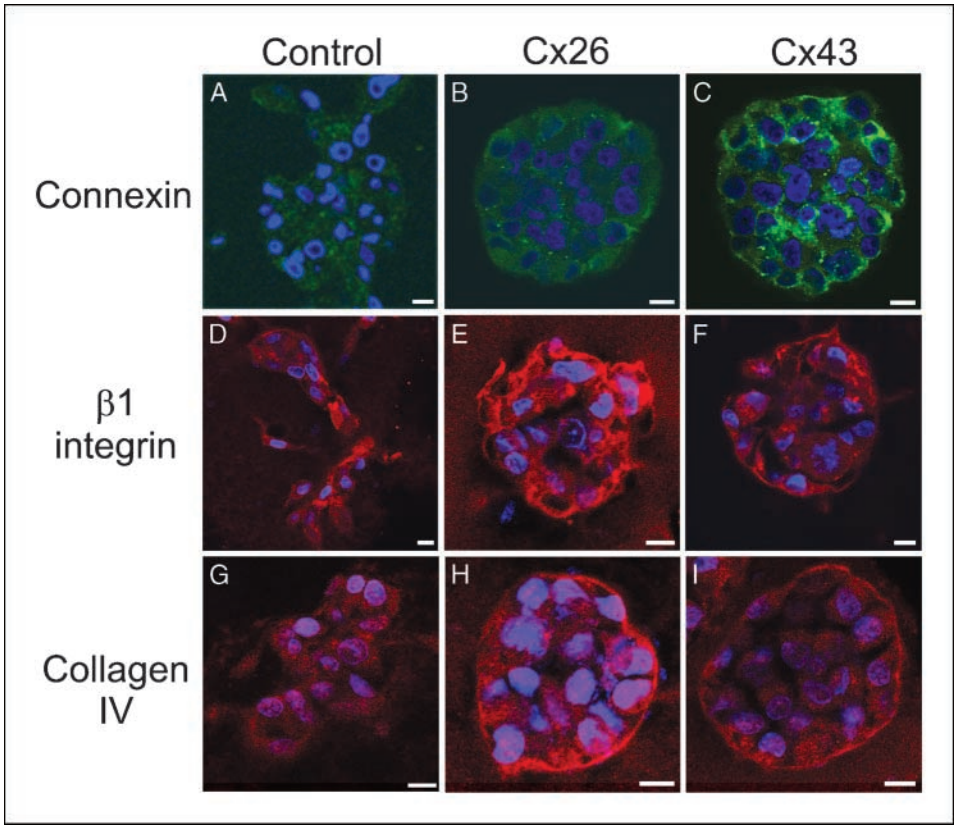

Figure 2. Connexin overexpression does not significantly increase gap junction plaque formation in organoids but induces organoid polarity. Three-dimensional cultures were immunolabeled to reveal the spatial localization of Cx26 (*B*) or Cx43 (*A* and *C; green*), β_1 integrin (*D-F*), (*red*) and type IV collagen (*G-I; red*). Cell nuclei were counterstained with Hoechst 33342 (*blue*). Connexin-overexpressing organoids were spherical with only a minor subpopulation of connexins localized to punctate structures at cell-cell interfaces. β_1 integrin and type IV collagen typically showed a diffuse localization pattern in control, stellate organoids but localized to the basal surface of connexin-expressing, spherical organoids. Bar, 10 μm.

stellate structures typical of malignant cells; however, cells expressing either Cx26 or Cx43 grew as spherical organoids more reminiscent of the acinar growth of normal mammary epithelial cells (Fig. 1*B*). The spherical structures localized both β_1 integrin and type IV collagen to the basal surface of the cell, whereas control organoids showed a more diffuse pattern of localization (Fig. 2). However, the spherical organoids were of heterogeneous size and did not form a lumen indicating that the overexpression of Cx26 or Cx43 induces only partial redifferentiation (Fig. 2). These findings are consistent with connexin-mediated three-dimensional growth and partial redifferentiation of MDA-MB-435 cells as previously reported (7). However, in MDA-MB-435 cells, it was assumed that the mechanism required for this redifferentiation was due to GJIC and not merely the presence of the connexins.

Connexins rarely assemble into detectable gap junctions at the cell-cell interface and do not reestablish GJIC in three-dimensional cultures as assessed by dye transfer. Immunocytochemistry of the organoids in three-dimensional culture revealed that overexpressed Cx26 or Cx43 did not seem to significantly increase the number of gap junction plaques at the cell surface (Fig. 2). Nevertheless, we tested whether GJIC was restored by the overexpressed connexins by fluorescence recovery after photobleaching (FRAP). Regardless of whether the organoids overexpressed Cx26 or Cx43, FRAP revealed no evidence that functional gap junction channels had formed (Fig. 3). As a positive control, fluorescent dye readily spread into the photobleached areas of connexin-positive MCF10A cells. These studies suggest that the connexin-induced partial reversion of MDA-MB-231 cells to a less malignant phenotype is independent of GJIC.

Connexin overexpression reduces cell migration and promotes a phenotypic mesenchymal to epithelial shift. We theorized that the expression of connexins may play a role in reversing the process of epithelial to mesenchymal transition (EMT) normally required for carcinogenic transformation. Because EMT is linked to cell migration (18), we first plated connexin-overexpressing cells on top of a Transwell filter, and migration through the pores to the bottom of the well was assessed after 24 hours. MDA-MB-231 cells overexpressing either Cx26 or Cx43 exhibited over 75% less incidence of migration through the filters compared with control cells (Fig. 4*A*). Similarly, a mildly aggressive variant of MDA-MB-435 cells expressing functional and nonfunctional Cx26 showed reduced migration potential compared with controls, whereas cells expressing Cx43 did not (16), suggesting some cell type differences.

The ratio of the intermediate filament proteins cytokeratin 18 to vimentin is often used to assess the phenotypic properties of cells that have undergone EMT (18). Three-dimensional cultures of connexin-expressing MDA-MB-231 cells showed a significant reduction in vimentin levels compared with control cells (Fig. 4*B* and *C*). Conversely, the expression of cytokeratin 18 was found to be very low in three-dimensional cultures of control MDA-MB-231 cells but reexpressed at high levels in connexin-expressing cells (Fig. 4*B* and *D*). When expression of theses molecules was

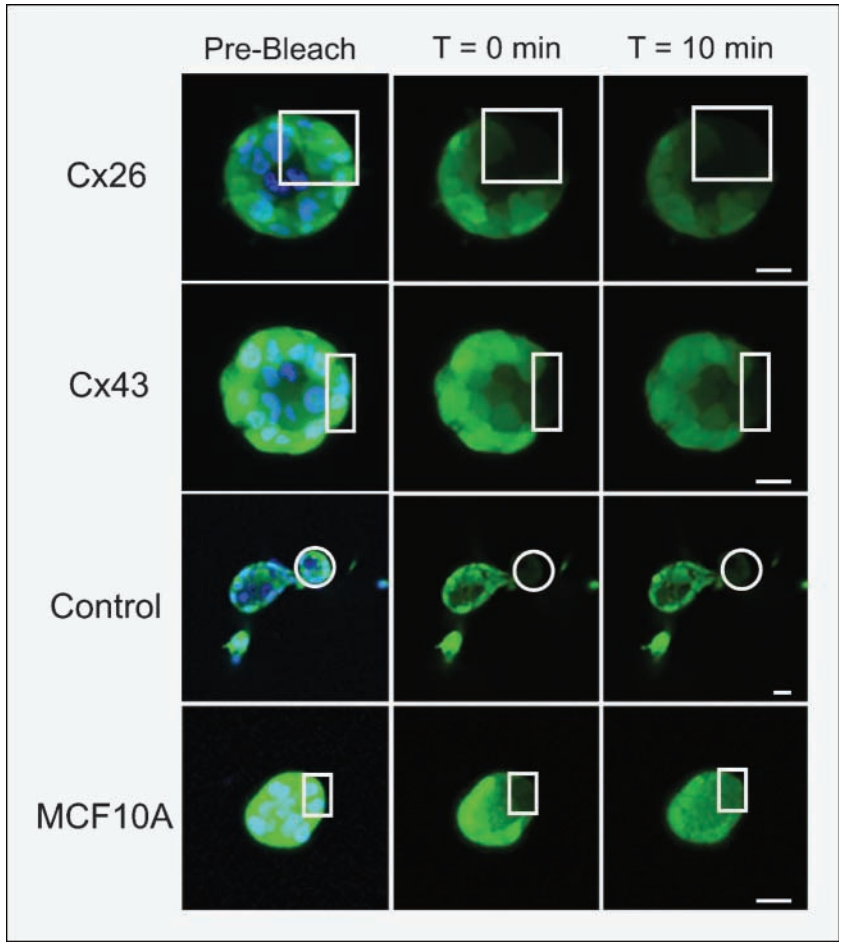

Figure 3. Connexins in three-dimensional organoids are nonfunctional. FRAP analysis revealed dye recovery into the photobleached area of MCF10A cells, a GJIC-competent cell line, whereas neither the control or connexin-overexpressing MDA-MB-231 cells revealed dye spread back into the photobleached area. Cell nuclei were stained with Hoechst 33342. Bar, 20 μm.

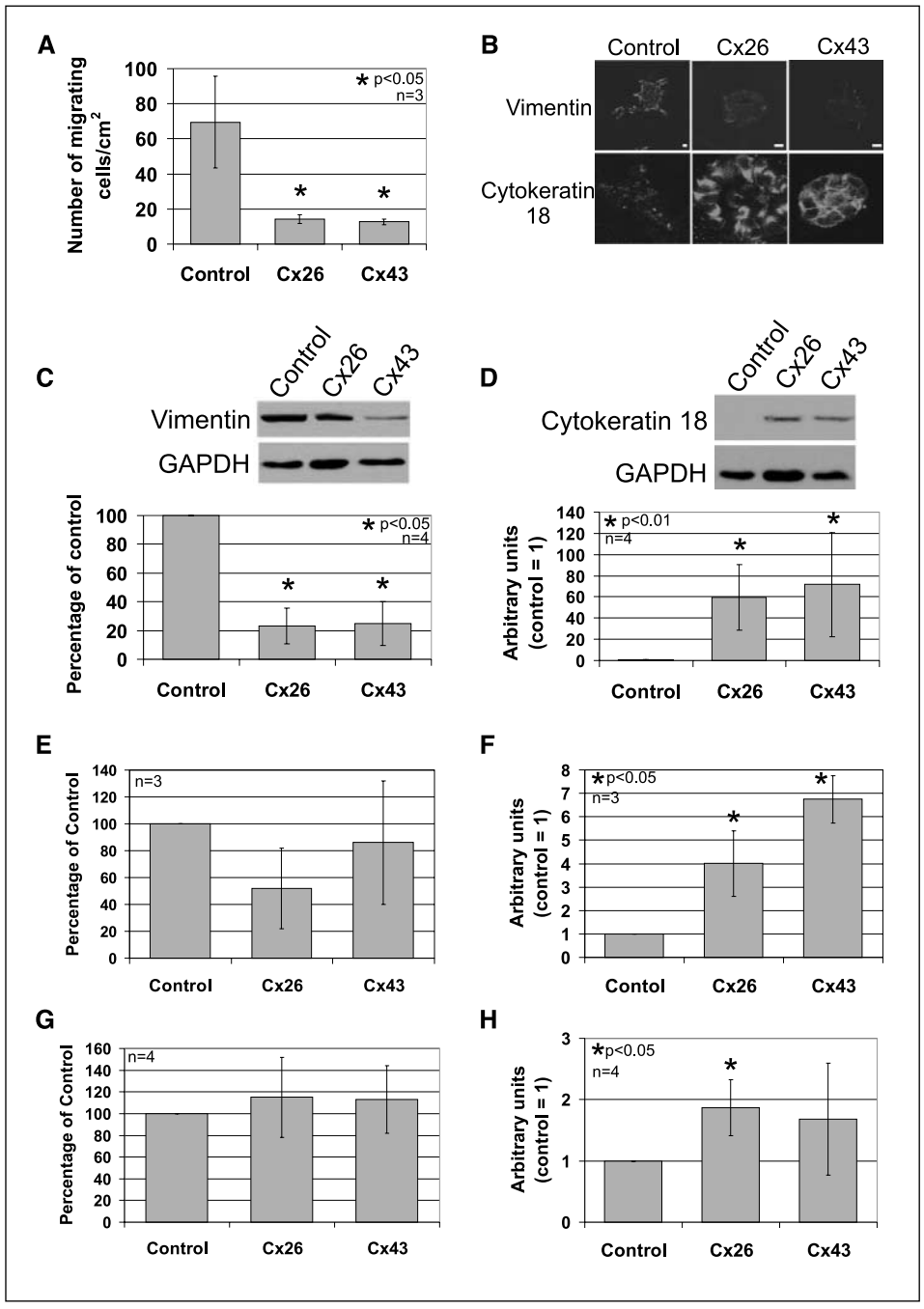

Figure 4. Connexin overexpression promotes mesenchymal to epithelial redifferentiation. *A,* cells overexpressing either Cx26 or Cx43 showed significantly less migration than control cells. *B,* immunolocalization studies revealed that three-dimensional organoids overexpressing either Cx26 or Cx43 have significantly reduced levels of vimentin, whereas they show significantly increased levels of cytokeratin 18 compared with controls. This is confirmed by Western blot quantification of vimentin (*C*) and cytokeratin 18 (*D*). Cytokeratin 18 was also up-regulated in two-dimensional cultures of MDA-MB-231 (*F*) and MDA-MB-435 (*H*) cells overexpressing Cx26 or Cx43, although to a lesser degree than seen in three-dimensional cultures. In contrast, regulation of vimentin was not evident in two-dimensional cultures of MDA-MB-231 (*E*) or MDA-MB-435 (*G*) cells. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal gel loading control.

examined in the two-dimensional cultures of MDA-MB-231 cells, the effect on cytokeratin 18 expression was much less pronounced, although still statistically significant, and there was no effect on vimentin expression (Fig. 4*E* and *F*), indicating that a three-dimensional milieu is essential to fully appreciate connexin-mediated modes of tumor suppression. Similarly, the expression levels of cytokeratin 18 were only slightly increased with connexin expression in two-dimensional cultures of MDA-MB-435 cells, whereas no change in vimentin levels was seen (Fig. 4*G* and *H*). Collectively, these studies support the conclusion that connexin expression partially reverts the transformed phenotype of breast cancer cells by regulating EMT, which is particularly evident in three-dimensional culture.

Connexins regulate the expression of molecules important in angiogenesis and reduces endothelial cell tubulogenesis and migration. Because connexins often exhibit their tumor-suppressive properties more clearly within the three-dimensional milieu of an *in vivo* environment, we speculated that they may be regulating processes involved in angiogenesis. To that end, we probed a human angiogenesis antibody array with conditioned media collected from three-dimensional cultures of control versus connexin-expressing MDA-MB-231 cells. Monocyte chemotactic protein-1 (MCP-1) and IL-6 were consistently up-regulated by both Cx26 and Cx43 expression (Fig. 5*A* and *B*). Semiquantitative RT-PCR suggests that the connexin-mediated regulation of MCP-1 and IL-6 occurs at the transcriptional level as the mRNA levels

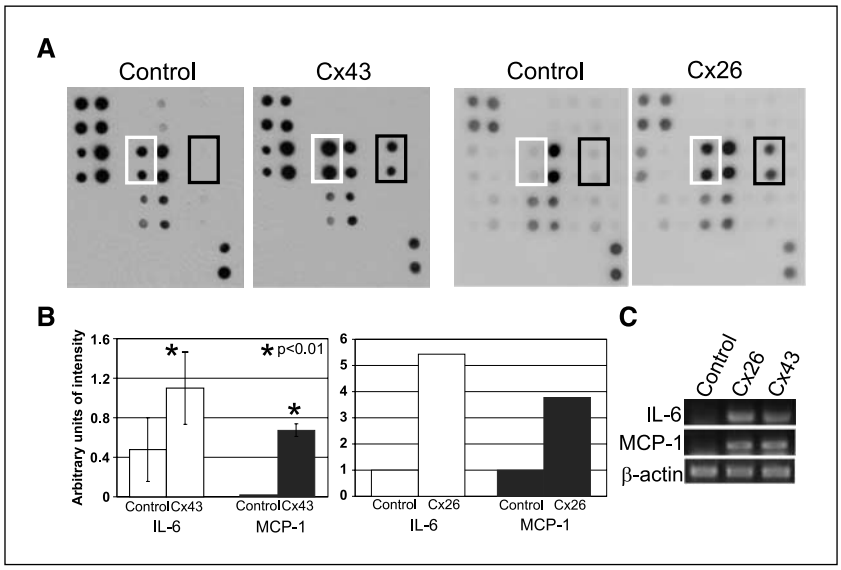

Figure 5. Connexin overexpression regulates angiogenesis genes. A RayBio Human Angiogenesis Antibody Array (RayBiotech) was incubated with conditioned media recovered from three-dimensional cultures of control or Cx43- or Cx26-overexpressing MDA-MB-231 cells. *A,* both Cx43 and Cx26 overexpression resulted in up-regulation of IL-6 (*white*) and MCP-1 (*black*). *B,* densitometry analysis of IL-6 and MCP-1 signals were normalized to positive and negative antibody array controls for Cx43 ($n = 4$) and Cx26 ($n = 2$). *C,* RT-PCR analysis showed transcriptional up-regulation of both IL-6 and MCP-1 in connexin-overexpressing MDA-MB-231 cells.

seem up-regulated in three-dimensional cultures of connexin-expressing MDA-MB-231 cells compared with controls (Fig. 5*C*).

To assess the functional consequence of the regulation of molecules important to angiogenesis, endothelial tubulogenesis and migration assays were carried out in a manner that would evaluate if the overexpression of connexins would inhibit tubulogenesis. Endothelial cells were grown atop a thin layer of Matrigel and allowed to form networks of tube-like structures indicative of their angiogenic ability. When the endothelial cells were grown in conditioned medium recovered from either two-dimensional or three-dimensional cultures of connexin-expressing breast cancer cells, tubular network formation was significantly reduced compared with that of endothelial cells grown in medium from control cells (Fig. 6*A, B, D*, and *F*). In addition, the migration of endothelial cells was reduced in the presence of conditioned medium recovered from cultures of either Cx26- or Cx43-expressing breast cancer cells compared with conditioned medium from control cells (Fig. 6*C, E*, and *G*).

Cx43-overexpressing tumor xenoplants in nude mice have reduced numbers of blood vessels. We have previously shown that Cx26- and Cx43-overexpressing MDA-MB-231 cells are less tumorigenic in nude mice than MDA-MB-231 control cells (13). We reexamined the xenoplant tumors and found that there were ~25% less vessels found in tissue sections taken from xenoplant tumors that overexpressed Cx43 compared with the control group (Fig. 6*H*). Collectively, these *in vitro* and *in vivo* studies suggest that connexin expression stimulates the release of angiogenesis inhibitors or inhibits the secretion of proangiogenic factors, resulting in a balance shifting towards inhibition of angiogenic processes.

Discussion

We have shown that both Cx26 and Cx43 can act as tumor suppressors, and that many of their effects are more pronounced in a three-dimensional environment. Overexpression of either of these connexins in normally very aggressive breast cancer cells inhibited their malignant properties. Significantly, connexin expression reduced anchorage-independent growth and allowed the partial redifferentiation of MDA-MB-231 three-dimensional organoid growth despite not appreciably increasing the formation of gap junction plaques or rescuing gap junction-mediated dye transfer. This finding provided us with a model to further examine the GJIC-independent tumor-suppressive roles of connexins. Two aspects of tumorigenesis were identified to be affected by connexin-dependent mechanisms. First, expression of either Cx26 or Cx43 seemed to partially revert the EMT associated with cell transformation. Second, an angiogenesis antibody array, tubulogenesis and migration assays, and analysis of vessel formation in xenoplant tumors all point to an angiogenesis-related mechanism of connexin-mediated tumor suppression.

Three-dimensional culture systems are essential for understanding the role of connexins in mammary differentiation and tumorigenesis. Using three-dimensional cultures of immortal normal mouse CID-9 mammary cells, El-Sabban et al. (19) showed that, upon incubation in a reconstituted basement membrane matrix, Cx26, Cx32, and Cx43 localized to the plasma membrane, and GJIC was enhanced. Furthermore, blocking gap junction function with 18α-glycyrrhetinic acid in CID-9 cells resulted in a decrease in β-casein secretion, indicative of reduced differentiation. Reciprocally, cyclic AMP induced up-regulation of Cx26 and Cx43 expression and overall GJIC initiated differentiation. In the human mammary carcinoma cell line MDA-MB-435 cells, exogenous expression of Cx26 or Cx43 restored GJIC and the capacity of the cells to form three-dimensional structures in reconstituted extracellular matrix (7). In our studies using MDA-MB-231 cells, three-dimensional organoids recapitulated the tumor-suppressive properties of connexins seen only *in vivo* xenoplants, highlighting the importance of cell-matrix interactions and organoid architecture. Importantly, we extended previous understandings to show that the partial reversion of the tumor cells was independent of GJIC as assessed by dye transfer.

Connexins act as tumor suppressors in a GJIC-independent manner. It remains controversial as to whether the mechanism by which connexins act as tumor suppressors is due to increased intercellular exchange of small molecules or due to an alternate connexin-dependent mechanism. Several studies have reported suggestive evidence that tumor-suppressive effects of connexin expression are GJIC-independent (13, 20–24). A GJIC-independent mechanism of tumor suppression is supported in our study where a

partial reversion of the malignant characteristics of MDA-MB-231 cells is observed in three-dimensional growth environments without rescue of dye transfer. Furthermore, the expression of nonfunctional Cx26 in MDA-MB-435 cells has the same effect as functional Cx26 compared with control cells on inhibiting the tumorigenic properties of MDA-MB-435 cells, including anchorage-independent growth and cell migration (16).

One mechanism by which connexins may act to regulate cell growth and differentiation in a GJIC-independent manner is through transient binding partners that signal and regulate cell growth and carcinogenesis. We know now that Cx43 binds directly or indirectly to many partners that include ZO-1 (25–27), tubulin (28), caveolin 1 (29), catenins (30), CCN3/NOV (31, 32), CIP85 (33), and drebrin (34), whereas Cx26 has been shown to bind to the OCP1 subunit of the E3 ubiquitin ligase (35) and caveolin-1 (29). Several of these connexin-binding proteins have potential roles in maintaining cytoarchitecture and tumorigenesis. Therefore, in many breast tumor cells, misregulation of connexins may lead to dysregulated interactions with binding proteins, affecting several tumorigenesis pathways.

Connexins regulate molecular pathways linked to EMT and angiogenesis. To begin to assess the down-stream molecules regulated by connexin expression in MDA-MB-231 cells that may be related to the connexin-based mechanisms of tumor suppression, we examined two separate processes of tumorigenesis: EMT and angiogenesis. We showed that expression of Cx43 or Cx26 results in increased cytokeratin 18 levels in the mammary epithelial cells, whereas vimentin levels decreased, and the cells were less migratory. Both of these cytoskeletal proteins are indicators for EMT, and epithelial cells are known to have less migratory potential than mesenchymal cells (18). A connexin-mediated shift towards a more epithelial cell phenotype was also confirmed by the up-regulation of cytokeratin-18 in connexin-expressing MDA-MB-435 cells. Full reversion to an epithelial phenotype, however, would include restoration of cell polarization (reviewed in ref. 36), which is not evident in our MDA-MB-231 three-dimensional organoids. Although organoids overexpressing Cx26 or Cx43 localized β_1 integrin and type IV collagen to the basal surface of the cell, the spherical organoids did not seem to growth arrest or form a lumen indicating only a partial reversion to an epithelial phenotype. EMT is a multistep process that is hypothesized to be highly influenced by the tissue microenvironment (37, 38). It is, therefore, not surprising that connexin expression alone does not allow the complete reversion of mammary tumor cells to an epithelial phenotype but suggests that connexins may be an essential component in this process.

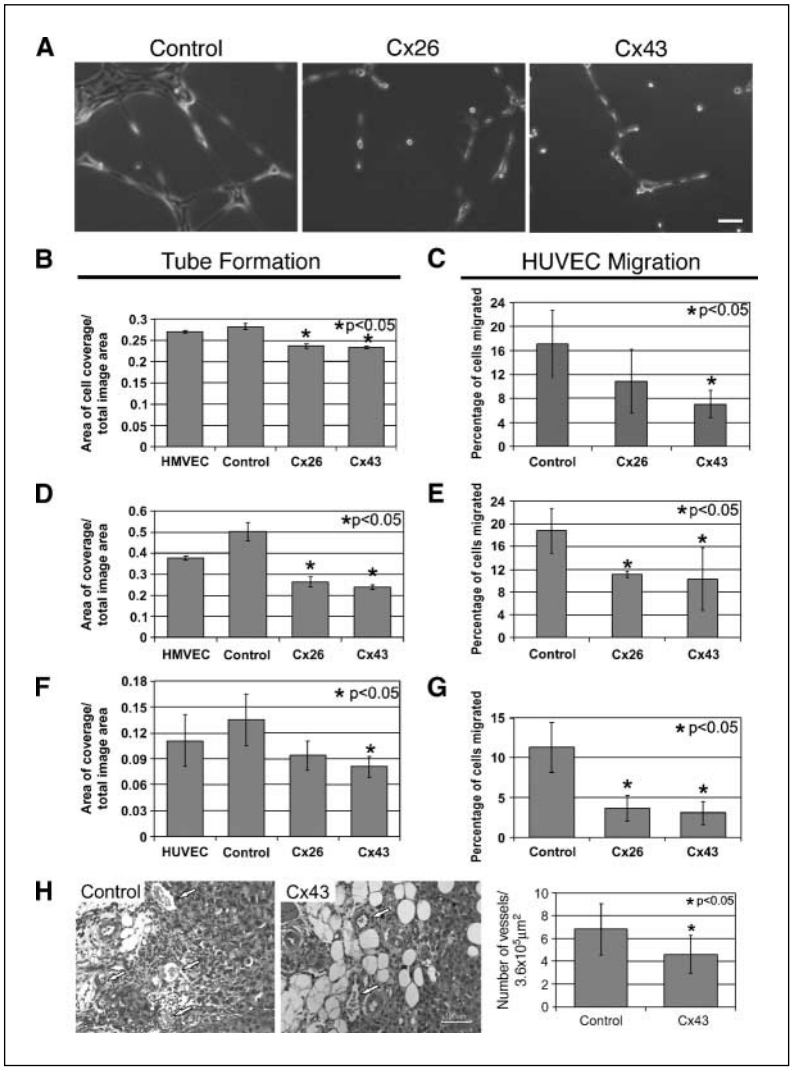

Figure 6. Connexin-overexpressing cells secrete factors that inhibit endothelial cell tube formation and migration *in vitro* and reduce tumor vascularization *in vivo*. *A,* differential formation of endothelial tube-like structures in the presence of conditioned media recovered from three-dimensional cultures of control or Cx26- or Cx43-expressing MDA-MB-231 cells. Bar, 50 μm. Tube formation was reduced in the presence of conditioned media recovered from breast cancer cells overexpressing either Cx26 or Cx43 compared with cells grown in conditioned media from viral control cells (Control; *B, D*, and *F*). Conditioned medium was assayed from three-dimensional cultures of MDA-MB-231 cells (*B*), two-dimensional cultures of MDA-MB-231 cells (*D*), or two-dimensional cultures of MDA-MB-435 cells (*F*). Endothelial cells were also grown in serum-containing endothelial cell media as a control for induction of tube formation. Total image area = 1.58×10^5 μm^2. Migration of HUVEC cells plated on top of a Transwell filter was inhibited in the presence of conditioned media from connexin-overexpressing cells on the other side of the filter compared with conditioned media from control cells for three-dimensional cultures of MDA-MB-231 cells (*C*), two-dimensional cultures of MDA-MB-231 cells (*E*), or two-dimensional cultures of MDA-MB-435 cells (*G*). Experiments were repeated three or four times. Bar, 50 μm. *H,* the number of vessels (*arrows*) found in tumor areas were calculated from sections of xenoplants of control or Cx43-overexpressing MDA-MB-231 tumors formed in the mammary fat pad of nude mice (13). Cx43-expressing tumors form significantly less vessels than controls.

Angiogenesis is a key factor for tumor viability and growth (39). We previously showed that Cx26 expression in MDA-MB-435 breast tumor cells resulted in increased transcription and translation of TSP-1, an antiangiogenic factor (14). Moreover, a nonfunctional GFP-Cx26 chimera also increased TSP-1 expression, indicating that the mechanism of regulation was not GJIC-dependent. Similarly, small interfering RNA silencing of Cx43 in Hs578T breast tumor cells resulted in decreased expression of TSP-1 and increased vascular endothelial growth factor expression, a proangiogenic molecule (40). In our present study, IL-6 and MCP-1 were up-regulated by Cx43 expression. This contrasts with previous findings that Cx26 and Cx43 expression down-regulated MCP-1 levels in glioblastoma cells, leading to suppressed growth (41), and may suggest some cell type differences. Similarly, there have been many reports positively correlating expression levels of IL-6 and connexins as a part of the inflammatory response (42–45); however, these all propose IL-6-mediated up-regulation of connexins rather than the connexin-mediated regulation of IL-6 as seen in this study. Our results indicate that not all proangiogenic or antiangiogenic molecules are regulated in synergy, which highlights the complexity of connexin-mediated pathways that likely are involved in cell growth and differentiation under various stimuli and in distinct cell types.

Here, we provide the first direct evidence that connexins play a role in regulating angiogenesis in breast tumors. To assess the balance between proangiogenic and antiangiogenic factors, we employed *in vitro* assays examining endothelial cell tube formation and migration, which revealed a connexin-mediated shift towards inhibition of these angiogenic processes in two different breast cancer cell lines. These findings were supported *in vivo* by analysis of MDA-MB-231 xenoplant tumors, which revealed that tumors with up-regulated Cx43 expression are less vascularized.

In summary, we have shown that Cx26 and Cx43 act similarly in human MDA-MB-231 cells to inhibit tumorigenesis. The mechanism is GJIC-independent but connexin-dependent and seems to involve the downstream regulation of factors governing EMT and angiogenesis. Furthermore, this study illustrates that three-dimensional organoid cultures are imperative to elucidate the role of connexins in breast carcinogenesis.

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